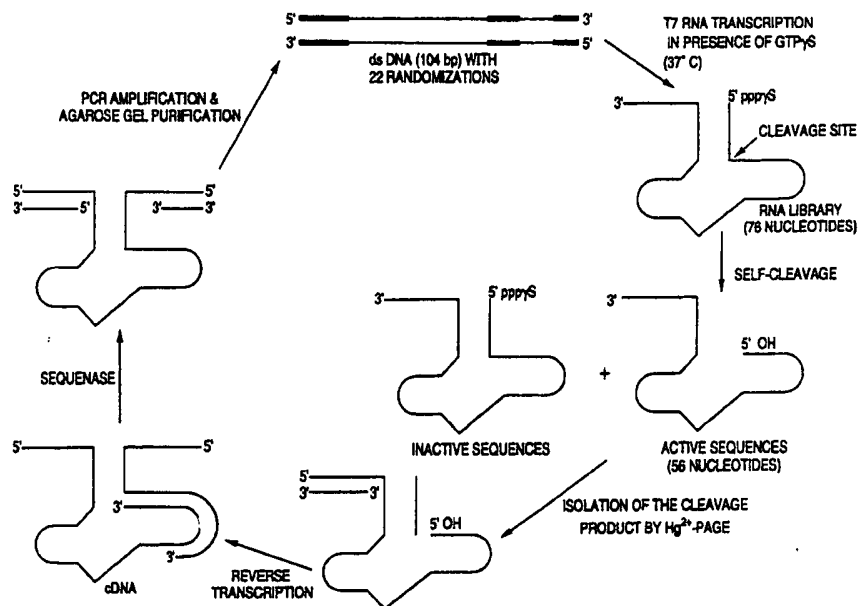




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/11, 9/00, C07H 21/02, A61K 31/70, C12Q 1/68 // C12N 15/55		A3	(11) International Publication Number: WO 99/16871
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(21) International Application Number: PCT/US98/19840		(74) Agents: WARBURG, Richard, J. et al.; Lyon & Lyon LLP, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).	
(22) International Filing Date: 22 September 1998 (22.09.98)			
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(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/059,437 (CON) Filed on 22 September 1997 (22.09.97)			
(71) Applicant (for all designated States except US): MAX-PLANCK-GESELLSCHAFT ZUR FORDERUNG DER WISSENSC [DE/DE]; HAFTEN E.V., Hofgarten-Strasse 2, D-80539 Munich (DE).			
(72) Inventors; and (75) Inventors/Applicants (for US only): ECKSTEIN, Fritz [DE/DE]; Holunderstog 6, D-37077 Gottingen (DE). HEATON, Paul, A. [GB/GB]; 27 Windsor Road, Golborne, Warrington, Cheshire WA3 3HS (GB). VAISH, Narendra, K. [IN/US]; Hermann-Rein-Strasse 3, D-37075 Gottingen (DE).			
		Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
		(88) Date of publication of the international search report: 20 May 1999 (20.05.99)	

(54) Title: NUCLEIC ACID CATALYSTS WITH ENDONUCLEASE ACTIVITY



(57) Abstract

Catalytic nucleic acids, i.e. ribozymes, with new motifs giving an altered endonuclease specificity, methods of screening, synthesis and expression, and uses thereof in modulating gene expression.

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 98/19840

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/11 C12N9/00 C07H21/02 A61K31/70 C12Q1/68
//C12N15/55

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K C07H C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TANG, JIN ET AL: "Examination of the catalytic fitness of the hammerhead ribozyme by in vitro selection" RNA (1997), 3(8), 914-925, XP002084354 cited in the application see the whole document ---	1-9, 11-23, 27-30
A	VAISH, N. ET AL.: "Isolation of Hammerhead Ribozymes with Altered Core Sequences by in Vitro Selection" BIOCHEMISTRY 36(21), 27 May 1997, pages 6495-6501, XP002097464 cited in the application see the whole document --- -/--	1-7, 11-23, 30, 31

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

23 March 1999

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/19840

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHRISTOFFERSEN R E ET AL: "RIBOZYMES AS HUMAN THERAPEUTIC AGENTS" JOURNAL OF MEDICINAL CHEMISTRY, vol. 38, no. 12, 9 June 1995, pages 2023-2037, XP002062054 cited in the application see the whole document ---	24-27
A	WO 97 10328 A (RIBOZYME PHARM INC ;DOWELANCO (US); ZWICK MICHAEL G (US); EDINGTON) 20 March 1997 see examples 16-18 see claims 73-76 ---	25, 31-34
A	BENSELER F ET AL: "HAMMERHEAD-LIKE MOLECULES CONTAINING NON-NUCLEOSIDE LINKERS ARE ACTIVE RNA CATALYSTS" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 115, 1993, page 8483/8484 XP000644539 see the whole document ---	10
P,X	VAISH, N. ET AL.: "In vitro selection of a purine nucleotide-specific hammerhead-like ribozyme" PROC. NATL. ACAD. SCI. U. S. A. , 95(5), March 1998, pages 2158-2162, XP002097465 see the whole document -----	3,6,7, 10-16, 20,30

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/19840

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 26 to 29 (as far as in vivo methods are concerned) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
See FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 98 /19840

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

- In claims 1 to 3, J and K are defined as having a length of 7 and 6 respectively. However, in the dependent claims 4 to 6, the length of the corresponding J's and K's are only of 6 and 5 which renders the definition of these elements obscure. For the purpose of the search all claimed possibilities have been taken into account.

- The reference of claim 31 to an '... expression vector of claim 19...' is inconsistent as claim 19 is concerning a cell. The reference as been interpreted and searched as being to claim 20.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/19840

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9710328 A	20-03-1997	AU 6761796 A	01-04-1997
		CA 2226728 A	20-03-1997
		CN 1196091 A	14-10-1998
		EP 0842286 A	20-05-1998
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/11, 9/00, C07H 21/02, A61K 31/70, C12Q 1/68 // C12N 15/55	A2	(11) International Publication Number: WO 99/16871 (43) International Publication Date: 8 April 1999 (08.04.99)
(21) International Application Number: PCT/US98/19840 (22) International Filing Date: 22 September 1998 (22.09.98) (30) Priority Data: 60/059,473 22 September 1997 (22.09.97) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/059,437 (CON) Filed on 22 September 1997 (22.09.97) (71) Applicant (for all designated States except US): MAX-PLANCK-GESELLSCHAFT ZUR FORDERUNG DER WISSENSC [DE/DE]; HAFTEN E.V., Hof- garten-Strasse 2, D-80539 Munich (DE). (72) Inventors; and (75) Inventors/Applicants (for US only): ECKSTEIN, Fritz [DE/DE]; Holunderstog 6, D-37077 Gottingen (DE). HEATON, Paul, A. [GB/GB]; 27 Windsor Road, Golborne, Warrington, Cheshire WA3 3HS (GB). VAISH, Narendra, K. [IN/US]; Hermann-Rein-Strasse 3, D-37075 Gottingen (DE).	(74) Agents: WARBURG, Richard, J. et al.; Lyon & Lyon LLP, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>	
(54) Title: NUCLEIC ACID CATALYSTS WITH ENDONUCLEASE ACTIVITY (57) Abstract Catalytic nucleic acids, i.e. ribozymes, with new motifs giving an altered endoclease specificity, methods of screening, synthesis and expression, and uses thereof in modulating gene expression.		

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DESCRIPTIONNUCLEIC ACID CATALYSTS WITH ENDONUCLEASE ACTIVITYBackground of the Invention

5 This invention relates to nucleic acid molecules with catalytic activity and derivatives thereof.

The following is a brief description of enzymatic nucleic acid molecules. This summary is not meant to be complete but is provided only for understanding of the invention that follows. This summary is not an admission that all of the work described below is prior art to the claimed invention.

10 Enzymatic nucleic acid molecules (ribozymes) are nucleic acid molecules capable of catalyzing one or more of a variety of reactions, including the ability to repeatedly cleave other separate nucleic acid molecules in a nucleotide base sequence-specific manner. Such enzymatic nucleic acid molecules can be used, for example, to target virtually any RNA transcript (Zaug *et al.*, 324, *Nature* 429 1986 ; Cech, 260 *JAMA* 15 3030, 1988; and Jefferies *et al.*, 17 *Nucleic Acids Research* 1371, 1989).

Because of their sequence-specificity, *trans*-cleaving enzymatic nucleic acid molecules show promise as therapeutic agents for human disease (Usman & McSwiggen, 1995 *Ann. Rep. Med. Chem.* 30, 285-294; Christoffersen and Marr, 1995 *J. Med. Chem.* 38, 2023-2037). Enzymatic nucleic acid molecules can be designed to 20 cleave specific RNA targets within the background of cellular RNA. Such a cleavage event renders the mRNA non-functional and abrogates protein expression from that RNA. In this manner, synthesis of a protein associated with a disease state can be selectively inhibited.

25 There are seven basic varieties of naturally-occurring enzymatic RNAs. Each can catalyze the hydrolysis of RNA phosphodiester bonds in *trans* (and thus can cleave other RNA molecules) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of a enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the 30 enzymatic nucleic acid first recognizes and then binds a target RNA through

complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

In addition, several *in vitro* selection (evolution) strategies (Orgel, 1979, *Proc. R. Soc. London*, B 205, 435) have been used to evolve new nucleic acid catalysts capable of catalyzing a variety of reactions, such as cleavage and ligation of phosphodiester linkages and amide linkages, (Joyce, 1989, *Gene*, 82, 83-87; Beaudry *et al.*, 1992, *Science* 257, 635-641; Joyce, 1992, *Scientific American* 267, 90-97; Breaker *et al.*, 1994, *TIBTECH* 12, 268; Bartel *et al.*, 1993, *Science* 261:1411-1418; Szostak, 1993, *TIBS* 17, 89-93; Kumar *et al.*, 1995, *FASEB J.*, 9, 1183; Breaker, 1996, *Curr. Op. Biotech.*, 7, 442).

The enzymatic nature of a ribozyme is advantageous over other technologies, since the effective concentration of ribozyme necessary to effect a therapeutic treatment is generally lower than that of an antisense oligonucleotide. This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme (enzymatic nucleic acid) molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base-pairing mechanism of binding, but also on the mechanism by which the molecule inhibits the expression of the RNA to which it binds. That is, the inhibition is caused by cleavage of the RNA target and so specificity is defined as the ratio of the rate of cleavage of the targeted RNA over the rate of cleavage of non-targeted RNA. This cleavage mechanism is dependent upon factors additional to those involved in base-pairing. Thus, it is thought that the specificity of action of a ribozyme is greater than that of antisense oligonucleotide binding the same RNA site.

The development of ribozymes that are optimal for catalytic activity would contribute significantly to any strategy that employs RNA-cleaving ribozymes for the purpose of regulating gene expression. The hammerhead ribozyme functions with a catalytic rate (k_{cat}) of $\sim 1 \text{ min}^{-1}$ in the presence of saturating (10 mM) concentrations of Mg^{2+} cofactor. However, the rate for this ribozyme in Mg^{2+} concentrations that are closer to those found inside cells (0.5 - 2 mM) can be 10- to 100-fold slower. In contrast, the RNase P holoenzyme can catalyze pre-tRNA cleavage with a k_{cat} of ~ 30

min⁻¹ under optimal assay conditions. An artificial 'RNA ligase' ribozyme has been shown to catalyze the corresponding self-modification reaction with a rate of ~100 min⁻¹. In addition, it is known that certain modified hammerhead ribozymes that have substrate binding arms made of DNA catalyze RNA cleavage with multiple turn-over rates that approach 100 min⁻¹. Finally, replacement of a specific residue within the catalytic core of the hammerhead with certain nucleotide analogues gives modified ribozymes that show as much as a 10-fold improvement in catalytic rate. These findings demonstrate that ribozymes can promote chemical transformations with catalytic rates that are significantly greater than those displayed *in vitro* by most natural self-cleaving ribozymes. It is then possible that the structures of certain self-cleaving ribozymes may not be optimized to give maximal catalytic activity, or that entirely new RNA motifs could be made that display significantly faster rates for RNA phosphoester cleavage.

An extensive array of site-directed mutagenesis studies have been conducted with the hammerhead ribozyme to probe relationships between nucleotide sequence and catalytic activity. These systematic studies have made clear that most nucleotides in the conserved core of the hammerhead ribozyme cannot be mutated without significant loss of catalytic activity. In contrast, a combinatorial strategy that simultaneously screens a large pool of mutagenized ribozymes for RNAs that retain catalytic activity could be used more efficiently to define immutable sequences and to identify new ribozyme variants. Although similar *in vitro* selection experiments have been conducted with the hammerhead ribozyme (Nakamaye & Eckstein, 1994, *Biochemistry* 33, 1271; Long & Uhlenbeck, 1994, *Proc. Natl. Acad. Sci.*, 91, 6977; Ishizaka *et al.*, 1995, *BBRC* 214, 403; Vaish *et al.*, 1997, *Biochemistry* 36, 6495), none of these efforts have successfully screened full-sized hammerhead ribozymes for all possible combinations of sequence variants that encompass the entire catalytic core.

The hammerhead ribozyme is one of the smallest ribozymes known and has thus attracted much attention for the study of structure-function relationships in catalytic RNAs as well as for its potential for the sequence-specific inhibition of gene expression (Usman *et al.*, *supra*). The hammerhead cleaves RNA sequence-specifically adjacent to the general triplet sequence NUX, where N is any nucleotide and X can be A, U or C. Cleavage behind a guanosine such as in GUG is very slow (4.3×10^{-5} min⁻¹) compared to the triplet substrate GUC (1 min⁻¹) (Baidya *et al.*, 1997, *Biochemistry* 36, 1108). Although the X-ray structure of this ribozyme has been solved and a mechanism proposed (Pley *et al.*, 1994 *Nature*, 372, 68; Scott *et al.*, 1996, *Science* 274, 2065), the

question of what determines its specificity for the NUX sequence is still largely unresolved. One way of obtaining an insight into this problem might be to compare sequences of hammerhead ribozymes with different triplet cleaving specificities. In previous publications it was demonstrated, by *in vitro* selection, that the native
 5 hammerhead sequence to cleave typical cleavage triplet, NUX, can not be altered (Nakamaye & Eckstein, 1994, *Biochemistry* 33, 1271; Long & Uhlenbeck, 1994, *Proc. Natl. Acad. Sci.*, 91, 6977; Ishizaka *et al.*, 1995, *BBRC* 214, 403; Vaish *et al.*, 1997, *Biochemistry* 36, 6495).

Tang *et al.*, 1997, *RNA* 3, 914, reported novel ribozyme sequences with
 10 endonuclease activity, where the authors used an *in vitro* selection approach to isolate these ribozymes.

The references cited above are distinct from the presently claimed invention since they do not disclose and/or contemplate the catalytic nucleic acid molecules of the instant invention.

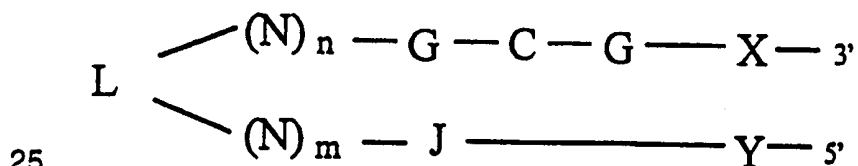
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Summary of the Invention

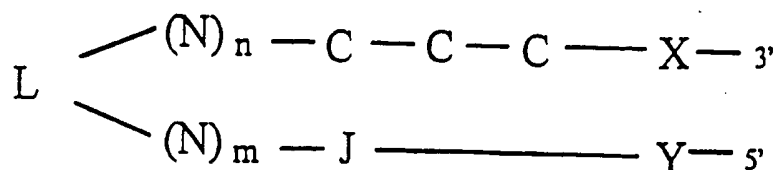
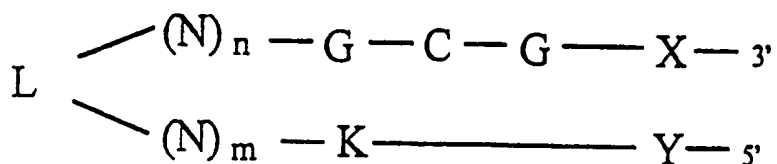
This invention relates to novel nucleic acid molecules with catalytic activity, which are particularly useful for cleavage of RNA or DNA. The nucleic acid catalysts of the instant invention are distinct from other nucleic acid catalysts known in the art. The nucleic acid catalysts of the instant invention do not share sequence homology with
 20 other known ribozymes. Specifically, nucleic acid catalysts of the instant invention are capable of catalyzing an intermolecular or intramolecular endonuclease reaction.

In a preferred embodiment, the invention features a nucleic acid molecule with catalytic activity having one of the formulae I-III:

Formula I



Formula II

Formula III

In each of the above formula, N represents independently a nucleotide or a non-nucleotide linker, which may be same or different; X and Y are independently oligonucleotides of length sufficient to stably interact (e.g., by forming hydrogen bonds with complementary nucleotides in the target) with a target nucleic acid molecule (the target can be an RNA, DNA or RNA/DNA mixed polymers); m and n are integers independently greater than or equal to 1 and preferably less than about 100, wherein if (N)_m and (N)_n are nucleotides, (N)_m and (N)_n are optionally able to interact by hydrogen bond interaction; J is an oligonucleotide of length 7 nucleotides; K is an oligonucleotide of length 6 nucleotides; L is a linker which may be present or absent (i.e., the molecule is assembled from two separate molecules), but when present, is a nucleotide and/or a non-nucleotide linker, which may be a single-stranded and/or double-stranded region; and _____ represents a chemical linkage (e.g. a phosphate ester linkage, amide linkage or others known in the art). C and G represent cytidine and guanosine nucleotides, respectively. The nucleotides in the each of the formula I-III are unmodified or modified at the sugar, base, and/or phosphate as known in the art.

In a preferred embodiment, the invention features nucleic acid molecules of Formula I, where the sequence of oligonucleotide J is selected from the group comprising 5'-GGCAUCC-3', 5'-AGCAUU-3', 5'-GCAUCA-3', 5'-AGCAUC-3', and 5'-AGCGUC-3'.

In another preferred embodiment, the invention features nucleic acid molecules of Formula II, where the sequence of oligonucleotide J is 5'-GGAAUC-3'.

In a further preferred embodiment, the invention features nucleic acid molecules of Formula III, where the sequence of oligonucleotide K is selected from the group comprising 5'-UGAUG-3', 5'-UGAUC-3', and 5'-UGGGC-3'.

5 In yet another embodiment, the nucleotide linker (L) is a nucleic acid aptamer, such as an ATP aptamer, HIV Rev aptamer (RRE), HIV Tat aptamer (TAR) and others (for a review see Gold *et al.*, 1995, *Annu. Rev. Biochem.*, 64, 763; and Szostak & Ellington, 1993, in *The RNA World*, ed. Gesteland and Atkins, pp 511, CSH Laboratory Press). A "nucleic acid aptamer" as used herein is meant to indicate nucleic acid sequence capable of interacting with a ligand. The ligand can be any natural or
10 synthetic molecule, including but not limited to a resin, metabolites, nucleosides, nucleotides, drugs, toxins, transition state analogs, peptides, lipids, proteins, amino acids, nucleic acid molecules, hormones, carbohydrates, receptors, cells, viruses, bacteria and others.

In yet another embodiment, the non-nucleotide linker (L) is as defined herein. The
15 term "non-nucleotide" as used herein include either abasic nucleotide, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, or polyhydrocarbon compounds. Specific examples include those described by Seela and Kaiser, *Nucleic Acids Res.* 1990, 18:6353 and *Nucleic Acids Res.* 1987, 15:3113; Cload and Schepartz, *J. Am. Chem. Soc.* 1991, 113:6324; Richardson and Schepartz, *J. Am. Chem. Soc.* 1991, 113:5109; Ma et al., *Nucleic Acids Res.* 1993, 21:2585 and *Biochemistry* 1993, 32:1751; Durand et al.,
20 *Nucleic Acids Res.* 1990, 18:6353; McCurdy et al., *Nucleosides & Nucleotides* 1991, 10:287; Jschke et al., *Tetrahedron Lett.* 1993, 34:301; Ono et al., *Biochemistry* 1991, 30:9914; Arnold *et al.*, International Publication No. WO 89/02439; Usman *et al.*, International Publication No. WO 95/06731; Dudycz *et al.*, International Publication
25 No. WO 95/11910 and Ferentz and Verdine, *J. Am. Chem. Soc.* 1991, 113:4000, all hereby incorporated by reference herein. Thus, in a preferred embodiment, the invention features an enzymatic nucleic acid molecule having one or more non-nucleotide moieties, and having enzymatic activity to cleave an RNA or DNA molecule. By the term "non-nucleotide" is meant any group or compound which can be incorporated into a nucleic
30 acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine. The

terms "abasic" or "abasic nucleotide" as used herein encompass sugar moieties lacking a base or having other chemical groups in place of base at the 1' position.

5 In preferred embodiments, the enzymatic nucleic acid includes one or more stretches of RNA, which provide the enzymatic activity of the molecule, linked to the non-nucleotide moiety. The necessary RNA components are known in the art (see for *e.g.*, Usman *et al.*, *supra*). By RNA is meant a molecule comprising at least one ribonucleotide residue.

10 Thus, in one preferred embodiment, the invention features ribozymes that inhibit gene expression and/or cell proliferation. These chemically or enzymatically synthesized nucleic acid molecules contain substrate binding domains that bind to accessible regions of specific target nucleic acid molecules. The nucleic acid molecules also contain domains that catalyze the cleavage of target. Upon binding, the enzymatic nucleic acid molecules cleave the target molecules, preventing for example, translation and protein accumulation. In the absence of the expression of the target gene, cell proliferation, for
15 example, is inhibited.

In another aspect of the invention, enzymatic nucleic acid molecules that cleave target molecules are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors are preferably DNA plasmids or viral vectors. Ribozyme expressing viral vectors could be constructed based on, but not limited to,
20 adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the recombinant vectors capable of expressing the ribozymes are delivered as described above, and persist in target cells. Alternatively, viral vectors may be used that provide for transient expression of ribozymes. Such vectors might be repeatedly administered as necessary. Once expressed, the ribozymes cleave the target mRNA. Delivery of
25 ribozyme expressing vectors could be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that would allow for introduction into the desired target cell (for a review see Couture and Stinchcomb, 1996, *TIG.*, 12, 510).

30 In a preferred embodiment, an expression vector comprising nucleic acid sequence encoding at least one of the nucleic acid catalyst of the instant invention is disclosed.

The nucleic acid sequence encoding the nucleic acid catalyst of the instant invention is operable linked in a manner which allows expression of that nucleic acid molecule.

In one embodiment, the expression vector comprises: a transcription initiation region (e.g., eukaryotic pol I, II or III initiation region); b) a transcription termination region (e.g., eukaryotic pol I, II or III termination region); c) a gene encoding at least one of the nucleic acid catalyst of the instant invention; and wherein said gene is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. The vector may optionally include an open reading frame (ORF) for a protein operably linked on the 5' side or the 3'-side of the gene encoding the nucleic acid catalyst of the invention; and/or an intron (intervening sequences).

By "patient" is meant an organism which is a donor or recipient of explanted cells or the cells themselves. "Patient" also refers to an organism to which enzymatic nucleic acid molecules can be administered. Preferably, a patient is a mammal or mammalian cells. More preferably, a patient is a human or human cells.

By "vectors" is meant any nucleic acid- and/or viral-based technique used to deliver a desired nucleic acid.

Another means of accumulating high concentrations of a ribozyme(s) within cells is to incorporate the ribozyme-encoding sequences into a DNA or RNA expression vector. Transcription of the ribozyme sequences are driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters will be expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type will depend on the nature of the gene regulatory sequences (enhancers, silencers, etc.) present nearby. Prokaryotic RNA polymerase promoters are also used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990 *Proc. Natl. Acad. Sci. USA*, 87, 6743-7; Gao and Huang 1993 *Nucleic Acids Res.*, 21, 2867-72; Lieber et al., 1993 *Methods Enzymol.*, 217, 47-66; Zhou et al., 1990 *Mol. Cell. Biol.*, 10, 4529-37). Several investigators have demonstrated that ribozymes expressed from such promoters can function in mammalian cells (e.g. Kashani-Sabet et al., 1992 *Antisense Res. Dev.*, 2, 3-15; Ojwang et al., 1992 *Proc. Natl. Acad. Sci. USA*, 89, 10802-6; Chen et al., 1992 *Nucleic Acids Res.*, 20, 4581-9; Yu et al., 1993 *Proc.*

Natl. Acad. Sci. U S A, 90, 6340-4; L'Huillier *et al.*, 1992 *EMBO J.* 11, 4411-8; Lisiewicz *et al.*, 1993 *Proc. Natl. Acad. Sci. U. S. A.*, 90, 8000-4; Thompson *et al.*, 1995 *Nucleic Acids Res.* 23, 2259; Sullenger & Cech, 1993, *Science*, 262, 1566). The above ribozyme transcription units can be incorporated into a variety of vectors for
5 introduction into mammalian cells, including but not restricted to, plasmid DNA vectors, viral DNA vectors (such as adenovirus or adeno-associated virus vectors), or viral RNA vectors (such as retroviral or alphavirus vectors) (for a review see Couture and Stinchcomb, 1996, *supra*).

In a preferred embodiment, the invention features a method of synthesis of
10 enzymatic nucleic acid molecules of instant invention which follows the procedure for normal chemical synthesis of RNA as described in Usman *et al.*, 1987 *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.*, 1990 *Nucleic Acids Res.*, 18, 5433; and Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-2684 and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and
15 phosphoramidites at the 3'-end. Small scale synthesis were conducted on a 394 Applied Biosystems, Inc. synthesizer using a modified 2.5 μ mol scale protocol with a 5 min coupling step for alkylsilyl protected nucleotides and 2.5 min coupling step for 2'-O-methylated nucleotides. Table I outlines the amounts, and the contact times, of the reagents used in the synthesis cycle. A 6.5-fold excess (163 μ L of 0.1 M = 16.3 μ mol)
20 of phosphoramidite and a 24-fold excess of S-ethyl tetrazole (238 μ L of 0.25 M = 59.5 μ mol) relative to polymer-bound 5'-hydroxyl is used in each coupling cycle. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, is 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer : detritylation
25 solution was 2% TCA in methylene chloride (ABI); capping was performed with 16% N-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution was 16.9 mM I₂, 49 mM pyridine, 9% water in THF (Millipore). B & J Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyl tetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained
30 from American International Chemical, Inc.

In a preferred embodiment, deprotection of the chemically synthesized nucleic acid catalysts of the invention is performed as follows. The polymer-bound oligoribonucleotide, trityl-off, is transferred from the synthesis column to a 4mL glass screw top vial and suspended in a solution of methylamine (MA) at 65 °C for 10 min.

After cooling to -20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H₂O/3:1:1, vortexed and the supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder.

- 5 The base-deprotected oligoribonucleotide is resuspended in anhydrous TEA•HF/NMP solution (250 µL of a solution of 1.5mL *N*-methylpyrrolidinone, 750 µL TEA and 1.0 mL TEA•3HF to provide a 1.4M HF concentration) and heated to 65°C for 1.5 h. The resulting, fully deprotected, oligomer is quenched with 50 mM TEAB (9 mL) prior to anion exchange desalting.
- 10 For anion exchange desalting of the deprotected oligomer, the TEAB solution is loaded on to a Qiagen 500® anion exchange cartridge (Qiagen Inc.) that is prewashed with 50 mM TEAB (10 mL). After washing the loaded cartridge with 50 mM TEAB (10 mL), the RNA is eluted with 2 M TEAB (10 mL) and dried down to a white powder. The average stepwise coupling yields are generally >98% (Wincott *et al.*, 1995
- 15 *Nucleic Acids Res.* 23, 2677-2684).

Ribozymes of the instant invention are also synthesized from DNA templates using bacteriophage T7 RNA polymerase (Milligan and Uhlenbeck, 1989, *Methods Enzymol.* 180, 51).

- 20 Ribozymes are purified by gel electrophoresis using general methods or are purified by high pressure liquid chromatography (HPLC; See Wincott *et al.*, *supra*) the totality of which is hereby incorporated herein by reference) and are resuspended in water.

- 25 In another preferred embodiment, catalytic activity of the molecules described in the instant invention can be optimized as described by Draper *et al.*, *supra*. The details will not be repeated here, but include altering the length of the ribozyme binding arms, or chemically synthesizing ribozymes with modifications (base, sugar and/or phosphate) that prevent their degradation by serum ribonucleases and/or enhance their enzymatic activity (see *e.g.*, Eckstein *et al.*, International Publication No. WO 92/07065; Perrault *et al.*, 1990 *Nature* 344, 565; Pieken *et al.*, 1991 *Science* 253, 314; Usman and Cedergren, 1992 *Trends in Biochem. Sci.* 17, 334; Usman *et al.*, International Publication No.
- 30 WO 93/15187; and Rossi *et al.*, International Publication No. WO 91/03162; Sproat, US Patent No. 5,334,711; and Burgin *et al.*, *supra*; all of these describe various chemical

modifications that can be made to the base, phosphate and/or sugar moieties of enzymatic RNA molecules). Modifications which enhance their efficacy in cells, and removal of bases from stem loop structures to shorten RNA synthesis times and reduce chemical requirements are desired. (All these publications are hereby incorporated by
5 reference herein.).

By "enhanced enzymatic activity" is meant to include activity measured in cells and/or *in vivo* where the activity is a reflection of both catalytic activity and ribozyme stability. In this invention, the product of these properties is increased or not significantly (less than 10 fold) decreased *in vivo* compared to an all RNA ribozyme.

10 In yet another preferred embodiment, nucleic acid catalysts having chemical modifications which maintain or enhance enzymatic activity is provided. Such nucleic acid is also generally more resistant to nucleases than unmodified nucleic acid. Thus, in a cell and/or *in vivo* the activity may not be significantly lowered. As exemplified herein such ribozymes are useful in a cell and/or *in vivo* even if activity over all is reduced 10
15 fold (Burgin *et al.*, 1996, *Biochemistry*, 35, 14090). Such ribozymes herein are said to "maintain" the enzymatic activity on all RNA ribozyme.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

20 The drawings will first briefly be described.

Drawings:

Figure 1 A) is a diagrammatic representation of the hammerhead ribozyme domain known in the art. Stem II can be ≥ 2 base-pair long. Each N is independently any base or non-nucleotide as used herein; B) is a diagrammatic representation of a self-cleaving
25 hammerhead ribozyme; C) is a diagrammatic representation of a random pool of self-cleaving RNA. N indicates the region with random nucleotides.

Figure 2 is a schematic representation of a non-limiting *in vitro* selection strategy used to evolve nucleic acid catalysts.

Figure 3 shows sequences of individual RNAs that represent new classes of self-cleaving ribozymes. Also shown are rates of catalysis (k_{in-cis} (min^{-1})) for each of the new self-cleaving RNA sequences. The underlined sequences indicate the regions capable of interacting with each other..

5 Figure 4 summarizes structure mapping studies on clone 40 RNA.

Figure 5 shows sequence and possible secondary structure of a trans-cleaving ribozyme of the invention. Arrow identifies the site of cleavage.

Figure 6 shows non-limiting examples of *trans*-cleaving ribozyme-substrate complex of the present invention.

10 Figure 7 shows non-limiting examples of chemically modified *trans*-cleaving ribozyme-substrate complex of the present invention.

Nucleic Acid Catalysts

15 The invention provides nucleic acid catalysts and methods for producing a class of enzymatic nucleic acid cleaving agents which exhibit a high degree of specificity for the nucleic acid sequence of a desired target. The enzymatic nucleic acid molecule is preferably targeted to a highly conserved sequence region of a target such that specific diagnosis and/or treatment of a disease or condition in a variety of biological system can be provided with a single enzymatic nucleic acid. Such enzymatic nucleic acid molecules can be delivered exogenously to specific cells as required. In the preferred hammerhead motif the small size (less than 60 nucleotides, preferably between 30-40 nucleotides in length) of the molecule allows the cost of treatment to be reduced.

20 By the phrase "nucleic acid catalyst" is meant a nucleic acid molecule capable of catalyzing (altering the velocity and/or rate of) a variety of reactions including the ability to repeatedly cleave other separate nucleic acid molecules (endonuclease activity) in a nucleotide base sequence-specific manner. Such a molecule with endonuclease activity may have complementarity in a substrate binding region (e.g. X and Y in formulae I-III) to a specified gene target, and also has an enzymatic activity that specifically cleaves RNA or DNA in that target. That is, the nucleic acid molecule with endonuclease activity is able to intramolecularly or intermolecularly cleave RNA or DNA and thereby
25 inactivate a target RNA or DNA molecule. This complementarity functions to allow
30

sufficient hybridization of the enzymatic RNA molecule to the target RNA or DNA to allow the cleavage to occur. 100% complementarity is preferred, but complementarity as low as 50-75% may also be useful in this invention. The nucleic acids may be modified at the base, sugar, and/or phosphate groups. The term enzymatic nucleic acid is used interchangeably with phrases such as ribozymes, catalytic RNA, enzymatic RNA, catalytic DNA, nucleozyme, DNazyme, RNA enzyme, endoribonuclease, endonuclease, minizyme, leadzyme, oligozyme or DNA enzyme. All of these terminologies describe nucleic acid molecules with enzymatic activity.

By "nucleic acid molecule" as used herein is meant a molecule comprising nucleotides. The nucleic acid can be composed of modified or unmodified nucleotides or non-nucleotides or various mixtures and combinations thereof.

By "complementarity" is meant a nucleic acid that can form hydrogen bond(s) with other RNA sequence by either traditional Watson-Crick or other non-traditional types (for example, Hoogsteen type) of base-paired interactions.

By "oligonucleotide" as used herein, is meant a molecule comprising two or more nucleotides.

The specific enzymatic nucleic acid molecules described in the instant application are not limiting in the invention and those skilled in the art will recognize that all that is important in an enzymatic nucleic acid molecule of this invention is that it has a specific substrate binding site (*e.g.*, X and Y of Formulae I-III above) which is complementary to one or more of the target nucleic acid regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart a nucleic acid cleaving activity to the molecule.

By "enzymatic portion" is meant that part of the ribozyme essential for cleavage of an RNA substrate.

By "substrate binding arm" or "substrate binding domain" is meant that portion of a ribozyme which is complementary to (*i.e.*, able to base-pair with) a portion of its substrate. Generally, such complementarity is 100%, but can be less if desired. For example, as few as 10 nucleotides out of a total of 14 may be base-paired. Such arms are shown generally in Figure 1A and as X and Y in Formulae I-III. That is, these arms contain sequences within a ribozyme which are intended to bring ribozyme and target

RNA together through complementary base-pairing interactions. The ribozyme of the invention may have binding arms that are contiguous or non-contiguous and may be of varying lengths. The length of the binding arm(s) are preferably greater than or equal to four nucleotides; specifically 12-100 nucleotides; more specifically 14-24 nucleotides long. The two binding arms are chosen, such that the length of the binding arms are symmetrical (*i.e.*, each of the binding arms is of the same length; *e.g.*, five and five nucleotides, six and six nucleotides or seven and seven nucleotides long) or asymmetrical (*i.e.*, the binding arms are of different length; *e.g.*, six and three nucleotides; three and six nucleotides long; four and five nucleotides long; four and six nucleotides long; four and seven nucleotides long; and the like).

Therapeutic ribozymes must remain stable within cells until translation of the target RNA has been inhibited long enough to reduce the levels of the undesirable protein. This period of time varies between hours to days depending upon the disease state. Clearly, ribozymes must be resistant to nucleases in order to function as effective intracellular therapeutic agents. Improvements in the chemical synthesis of RNA (Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677; incorporated by reference herein) have expanded the ability to modify ribozymes to enhance their nuclease stability. The term "nucleotide" is used as recognized in the art to include natural bases, and modified bases well known in the art. Such bases are generally located at the 1' position of a sugar moiety. Nucleotide generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (see for example, Usman and McSwiggen, *supra*; Eckstein *et al.*, International PCT Publication No. WO 92/07065; Usman *et al.*, International PCT Publication No. WO 93/15187; all hereby incorporated by reference herein). There are several examples of modified nucleic acid bases known in the art and has recently been summarized by Limbach *et al.*, 1994, *Nucleic Acids Res.* 22, 2183. Some of the non-limiting examples of base modifications that can be introduced into enzymatic nucleic acids without significantly effecting their catalytic activity include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyluracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (*e.g.*, 5-methylcytidine), 5-alkyluridines (*e.g.*, ribothymidine), 5-halouridine (*e.g.*, 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (*e.g.* 6-methyluridine) and others (Burgin *et al.*, 1996, *Biochemistry*, 35, 14090). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents;

such bases may be used within the catalytic core of the enzyme and/or in the substrate-binding regions.

In particular, the invention features modified ribozymes having a base substitution selected from pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy
5 benzene, 3-methyluracil, dihydrouracil, naphthyl, 6-methyl-uracil and aminophenyl.

There are several examples in the art describing sugar and phosphate modifications that can be introduced into enzymatic nucleic acid molecules without significantly effecting catalysis and with significant enhancement in their nuclease stability and efficacy. Ribozymes are modified to enhance stability and/or enhance catalytic activity
10 by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-fluoro, 2'-O-methyl, 2'-H, nucleotide base modifications (for a review see Usman and Cedergren, 1992 *TIBS* 17, 34; Usman *et al.*, 1994 *Nucleic Acids Symp. Ser.* 31, 163; Burgin *et al.*, 1996 *Biochemistry* 35, 14090). Sugar modification of enzymatic nucleic acid molecules have been extensively described in the art (see Eckstein *et al.*,
15 *International Publication* PCT No. WO 92/07065; Perrault *et al.* *Nature* 1990, 344, 565-568; Pieken *et al.* *Science* 1991, 253, 314-317; Usman and Cedergren, *Trends in Biochem. Sci.* 1992, 17, 334-339; Usman *et al.* *International Publication* PCT No. WO 93/15187; Sproat, *US Patent* No. 5,334,711 and Beigelman *et al.*, 1995 *J. Biol. Chem.* 270, 25702).

20 Such publications describe general methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into ribozymes without inhibiting catalysis, and are incorporated by reference herein. In view of such teachings, similar modifications can be used as described herein to modify the nucleic acid catalysts of the instant invention.

25 As the term is used in this application, non-nucleotide-containing enzymatic nucleic acid means a nucleic acid molecule that contains at least one non-nucleotide component which replaces a portion of a ribozyme, *e.g.*, but not limited to, a double-stranded stem, a single-stranded "catalytic core" sequence, a single-stranded loop or a single-stranded recognition sequence. These molecules are able to cleave (preferably,
30 repeatedly cleave) separate RNA or DNA molecules in a nucleotide base sequence specific manner. Such molecules can also act to cleave intramolecularly if that is desired. Such enzymatic molecules can be targeted to virtually any RNA transcript.

Administration of Ribozymes

Sullivan *et al.*, PCT WO 94/02595, describes the general methods for delivery of enzymatic RNA molecules. Ribozymes may be administered to cells by a variety of methods known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. For some indications, ribozymes may be directly delivered *ex vivo* to cells or tissues with or without the aforementioned vehicles. Alternatively, the RNA/vehicle combination is locally delivered by direct injection or by use of a catheter, infusion pump or stent. Other routes of delivery include, but are not limited to, intravascular, intramuscular, subcutaneous or joint injection, aerosol inhalation, oral (tablet or pill form), topical, systemic, ocular, intraperitoneal and/or intrathecal delivery. More detailed descriptions of ribozyme delivery and administration are provided in Sullivan *et al.*, *supra* and Draper *et al.*, PCT WO93/23569 which have been incorporated by reference herein.

The molecules of the instant invention can be used as pharmaceutical agents. Pharmaceutical agents prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state in a patient.

The negatively charged polynucleotides of the invention can be administered (*e.g.*, RNA, DNA or protein) and introduced into a patient by any standard means, with or without stabilizers, buffers, and the like, to form a pharmaceutical composition. When it is desired to use a liposome delivery mechanism, standard protocols for formation of liposomes can be followed. The compositions of the present invention may also be formulated and used as tablets, capsules or elixirs for oral administration; suppositories for rectal administration; sterile solutions; suspensions for injectable administration; and the like.

The present invention also includes pharmaceutically acceptable formulations of the compounds described. These formulations include salts of the above compounds, *e.g.*, acid addition salts, for example, salts of hydrochloric, hydrobromic, acetic acid, and benzene sulfonic acid.

A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, *e.g.*, systemic administration, into a cell or patient, preferably a human. Suitable forms, in part, depend upon the use or the

route of entry, for example oral, transdermal, or by injection. Such forms should not prevent the composition or formulation to reach a target cell (*i.e.*, a cell to which the negatively charged polymer is desired to be delivered to). For example, pharmacological compositions injected into the blood stream should be soluble. Other factors are known
5 in the art, and include considerations such as toxicity and forms which prevent the composition or formulation from exerting its effect.

By "systemic administration" is meant *in vivo* systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include, without
10 limitations: intravenous, subcutaneous, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. Each of these administration routes expose the desired negatively charged polymers, *e.g.*, nucleic acids, to an accessible diseased tissue. The rate of entry of a drug into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier comprising the compounds of the
15 instant invention can potentially localize the drug, for example, in certain tissue types, such as the tissues of the reticular endothelial system (RES). A liposome formulation which can facilitate the association of drug with the surface of cells, such as, lymphocytes and macrophages is also useful. This approach may provide enhanced delivery of the drug to target cells by taking advantage of the specificity of macrophage
20 and lymphocyte immune recognition of abnormal cells, such as the cancer cells.

The invention also features the use of the a composition comprising surface-modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes). These formulations offer an method for increasing the accumulation of drugs in target tissues. This class of drug carriers resists
25 opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug (Lasic *et al.* *Chem. Rev.* 1995, 95, 2601-2627; Ishiwata *et al.*, *Chem. Pharm. Bull.* 1995, 43, 1005-1011). Such liposomes have been shown to accumulate selectively in tumors, presumably by extravasation and capture in the neovascularized
30 target tissues (Lasic *et al.*, *Science* 1995, 267, 1275-1276; Oku *et al.*, 1995, *Biochim. Biophys. Acta*, 1238, 86-90). The long-circulating liposomes enhance the pharmacokinetics and pharmacodynamics of DNA and RNA, particularly compared to conventional cationic liposomes which are known to accumulate in tissues of the MPS (Liu *et al.*, *J. Biol. Chem.* 1995, 270, 24864-24870; Choi *et al.*, International PCT

Publication No. WO 96/10391; Ansell *et al.*, International PCT Publication No. WO 96/10390; Holland *et al.*, International PCT Publication No. WO 96/10392; all of these are incorporated by reference herein). Long-circulating liposomes are also likely to protect drugs from nuclease degradation to a greater extent compared to cationic liposomes, based on their ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen. All of these references are incorporated by reference herein.

The present invention also includes compositions prepared for storage or administration which include a pharmaceutically effective amount of the desired compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co. (A.R. Gennaro edit. 1985) hereby incorporated by reference herein. For example, preservatives, stabilizers, dyes and flavoring agents may be provided. *Id.* at 1449. These include sodium benzoate, sorbic acid and esters of *p*-hydroxybenzoic acid. In addition, antioxidants and suspending agents may be used.

A pharmaceutically effective dose is that dose required to prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state. The pharmaceutically effective dose depends on the type of disease, the composition used, the route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration, concurrent medication, and other factors which those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of active ingredients is administered dependent upon potency of the negatively charged polymer. In a one aspect, the invention provides enzymatic nucleic acid molecules that can be delivered exogenously to specific cells as required. The enzymatic nucleic acid molecules are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to smooth muscle cells. The RNA or RNA complexes can be locally administered to relevant tissues through the use of a catheter, infusion pump or stent, with or without their incorporation in biopolymers. Using the methods described herein, other enzymatic nucleic acid molecules that cleave target nucleic acid may be derived and used as described above. Specific examples of nucleic acid catalysts of the instant invention are provided below in the Tables and figures.

Alternatively, the enzymatic nucleic acid molecules of the instant invention can be expressed within cells from eukaryotic promoters (e.g., Izant and Weintraub, 1985 *Science* 229, 345; McGarry and Lindquist, 1986 *Proc. Natl. Acad. Sci. USA* 83, 399; Scanlon *et al.*, 1991, *Proc. Natl. Acad. Sci. USA*, 88, 10591-5; Kashani-Sabet *et al.*, 1992 5 *Antisense Res. Dev.*, 2, 3-15; Dropulic *et al.*, 1992 *J. Virol*, 66, 1432-41; Weerasinghe *et al.*, 1991 *J. Virol*, 65, 5531-4; Ojwang *et al.*, 1992 *Proc. Natl. Acad. Sci. USA* 89, 10802-6; Chen *et al.*, 1992 *Nucleic Acids Res.*, 20, 4581-9; Sarver *et al.*, 1990 *Science* 247, 1222-1225; Thompson *et al.*, 1995 *Nucleic Acids Res.* 23, 2259; Good *et al.*, 1997, *Gene Therapy*, 4, 45; all of the references are hereby incorporated in their totality by 10 reference herein). Those skilled in the art realize that any nucleic acid can be expressed in eukaryotic cells from the appropriate DNA/RNA vector. The activity of such nucleic acids can be augmented by their release from the primary transcript by a ribozyme (Draper *et al.*, PCT WO 93/23569, and Sullivan *et al.*, PCT WO 94/02595; Ohkawa *et al.*, 1992 *Nucleic Acids Symp. Ser.*, 27, 15-6; Taira *et al.*, 1991, *Nucleic Acids Res.*, 19, 5125-30; Ventura *et al.*, 1993 *Nucleic Acids Res.*, 21, 3249-55; Chowrira *et al.*, 15 1994 *J. Biol. Chem.* 269, 25856; all of the references are hereby incorporated in their totality by reference herein).

Transcription of the ribozyme sequences are driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase 20 III (pol III). Transcripts from pol II or pol III promoters will be expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type will depend on the nature of the gene regulatory sequences (enhancers, silencers, etc.) present nearby. Prokaryotic RNA polymerase promoters are also used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 25 1990 *Proc. Natl. Acad. Sci. U S A*, 87, 6743-7; Gao and Huang 1993 *Nucleic Acids Res.*, 21, 2867-72; Lieber *et al.*, 1993 *Methods Enzymol.*, 217, 47-66; Zhou *et al.*, 1990 *Mol. Cell. Biol.*, 10, 4529-37). Several investigators have demonstrated that ribozymes expressed from such promoters can function in mammalian cells (e.g. Kashani-Sabet *et al.*, 1992 *Antisense Res. Dev.*, 2, 3-15; Ojwang *et al.*, 1992 *Proc. Natl. Acad. Sci. U S A*, 89, 10802-6; Chen *et al.*, 1992 *Nucleic Acids Res.*, 20, 4581-9; Yu *et al.*, 1993 *Proc. Natl. Acad. Sci. U S A*, 90, 6340-4; L'Huillier *et al.*, 1992 *EMBO J.* 11, 4411-8; 30 Lisziewicz *et al.*, 1993 *Proc. Natl. Acad. Sci. U. S. A.*, 90, 8000-4; Thompson *et al.*, 1995 *Nucleic Acids Res.* 23, 2259; Sullenger & Cech, 1993, *Science*, 262, 1566). More specifically, transcription units such as the ones derived from genes encoding U6 small

nuclear (snRNA), transfer RNA (tRNA) and adenovirus VA RNA are useful in generating high concentrations of desired RNA molecules such as ribozymes in cells (Thompson *et al.*, *supra*; Couture and Stinchcomb, 1996, *supra*; Noonberg *et al.*, 1994, *Nucleic Acid Res.*, 22, 2830; Noonberg *et al.*, US Patent No. 5,624,803; Good *et al.*, 1997, *Gene Ther.* 4, 45; Beigelman *et al.*, International PCT Publication No. WO 96/18736; all of these publications are incorporated by reference herein. Examples of transcription units suitable for expression of ribozymes of the instant invention are shown in Figure 15. The above ribozyme transcription units can be incorporated into a variety of vectors for introduction into mammalian cells, including but not restricted to, plasmid DNA vectors, viral DNA vectors (such as adenovirus or adeno-associated virus vectors), or viral RNA vectors (such as retroviral or alphavirus vectors) (for a review see Couture and Stinchcomb, 1996, *supra*).

In yet another aspect the invention features an expression vector comprising nucleic acid sequence encoding at least one of the catalytic nucleic acid molecule of the invention, in a manner which allows expression of that nucleic acid molecule. The expression vector comprises in one embodiment; a) a transcription initiation region; b) a transcription termination region; c) a gene encoding at least one said nucleic acid molecule; and wherein said gene is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. In another preferred embodiment the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an open reading frame; d) a gene encoding at least one said nucleic acid molecule, wherein said gene is operably linked to the 3'-end of said open reading frame; and wherein said gene is operably linked to said initiation region, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. In yet another embodiment the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; d) a gene encoding at least one said nucleic acid molecule; and wherein said gene is operably linked to said initiation region, said intron and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. In another embodiment, the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; d) an open reading frame; e) a gene encoding at least one said nucleic acid molecule, wherein said gene is operably linked to the 3'-end of said open reading frame; and wherein said gene is operably linked to said initiation

region, said intron, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

By "consists essentially of" is meant that the active ribozyme contains an enzymatic center or core equivalent to those in the examples, and binding arms able to bind target nucleic acid molecules such that cleavage at the target site occurs. Other sequences may be present which do not interfere with such cleavage.

Examples

The following are non-limiting examples showing the selection, isolation, synthesis and activity of enzymatic nucleic acids of the instant invention.

10 An extensive array of site-directed mutagenesis studies have been conducted with the hammerhead to probe relationships between nucleotide sequence and catalytic activity. These systematic studies have made clear that most nucleotides in the conserved core of the hammerhead ribozyme (Forster & Symons, 1987, *Cell*, 49, 211) cannot be mutated without significant loss of catalytic activity. In contrast, a
15 combinatorial strategy that simultaneously screens a large pool of mutagenized ribozymes for RNAs that retain catalytic activity could be used more efficiently to define immutable sequences and to identify new ribozyme variants (Breaker, 1997, *supra*). For example, Joseph and Burke (1993; *J. Biol. Chem.*, 268, 24515) have used an *in vitro* selection approach to rapidly screen for sequence variants of the 'hairpin' self-cleaving RNA that show improved catalytic activity. This approach was successful in
20 identifying two mutations in the hairpin ribozyme that together give a 10-fold improvement in catalytic rate. Although similar *in vitro* selection experiments have been conducted with the hammerhead ribozyme (Nakamaye & Eckstein, 1994, *supra*; Long & Uhlenbeck, 1994, *supra*; Ishizaka et al., 1995, *supra*), none of these efforts have
25 successfully screened full-sized hammerhead ribozymes for all possible combinations of sequence variants that encompass the entire catalytic core.

Applicant employed *in vitro* selection strategy to comprehensively test whether the natural consensus sequence for the core of the hammerhead ribozyme produces maximal catalytic rates, or whether sequence variants of this ribozyme could catalyze
30 endonuclease reaction similar to or better than the hammerhead ribozyme.

The procedure reported herein to select for intramolecular AUG cleaving sequences is based on Applicant's previously reported selection of alternative GUC cleaving hammerhead sequences (Vaish *et al.*, *supra*; incorporated by reference herein).

Example 1: *In vitro* selection of self-cleaving RNAs from a random pool

5 It was of interest to see what changes have to be imposed on the native hammerhead sequence for it to cleave after AUG, which usually resists cleavage, and thus arrive at a ribozyme with a new specificity. To achieve this, an *in vitro* selection was undertaken, where the number of randomized positions in the starting pool corresponded to the number of nucleotides typically found in the core and stem-loop II
10 region of a hammerhead ribozyme. This would allow all possible sequence permutations to be explored in the search for a hammerhead which is able to cleave behind AUG (Robertson & Joyce, 1990; Ellington and Szostak, 1990; Tuerk & Gold, 1990; Wright & Joyce, 1997; Carmi *et al.*, 1997; for reviews see Breaker, 1997; Abelson, 1996; Ellington, 1997; Chapman & Szostak, 1994).

15 An initial pool of dsDNAs containing 22 randomized positions flanked by two constant regions were synthesized. These were transcribed, with transcription being initiated with GTP γ S, to produce a pool of potential ribozymes. Self-cleavage of active ribozymes occurs during transcription and the cleavage products can then be separated from intact transcripts by means of a mercury gel (Igloi, *Biochemistry* 27, 3842). The
20 recovered cleavage products were then reverse transcribed and amplified by PCR to give a dsDNA pool of selected ribozymes. This selection procedure was repeated for 13 cycles before the dsDNA pool was cloned and sequenced.

Oligodeoxyribonucleotides and oligoribonucleotides were chemically synthesized and purified as previously described (Vaish *et al.*, *supra*). Primers used in the selection:

25 **Primer 1:**

5'-

TGGTGCAAGCTTAATACGACTCACTATAGGGAGACTGTCTAGATCATGA
GGATGCTA-3';

Primer 2:

30 5'-

TCTCGGATCCTGCAGATCATNNNNNNNNNNNNNNNNNNNNNAGGATT
AGCATCCTCAT-3'.

These two primers were used for the initial Sequenase reaction, and Primer 2 also functioned as restoration primer for the reintroduction of the lost sequences during cleavage. Reverse transcription (RT)-Primer, 5'-TCTCGGATCCTGCAGATCAT-3'; and polymerase chain reaction (PCR)-Primer, 5'-
5 TGGTGCAGCTTAATACGACTCA-3', with HindIII (5'-end) and BamHI and PstI (3'-end) sites in boldface; T7 promoter is underlined; ribozyme cleavage triplet in italics; N, randomized nucleotides. RNA selection was performed as described (Vaish *et al.*, *supra*, incorporated by reference herein) with modifications to the initial two cycles of selection. Transcriptions from Pool 0 and Pool 1 were performed on a larger scale (10 x 500 μ l and 1 x 250 μ l, respectively) for 12 h and PCR amplification (Step 5) was
10 omitted in each case. Successive transcriptions were performed at 100 μ l volume. Transcription reactions were for decreasing periods of time from 12 h (1st cycle) to 1 min (13th cycle), where reactions for short periods were quenched by addition of EDTA (75 mM final conc.). All transcriptions were performed with 1 μ M DNA. DNA
15 template in the PCR mixture was at least 1×10^{-15} M. The minimum concentration of RNA template was 1×10^{-8} M for reverse transcription. The concentrations were determined assuming a molar extinction coefficient of 6600.

A selection pressure was exerted by progressively reducing the time of transcription from 12 h for the first six cycles, to 6 h for the seventh, 1 h for the eighth,
20 30 min for the ninth and tenth, 5 min for the eleventh, and 1 min for the twelve and thirteenth cycle. White colonies were selected randomly each from pools 7 and 10 and those showing self-cleavage upon transcription were sequenced. There was no homology among the sequences.

Cloning and sequencing was performed as described (Vaish *et al.*, *supra*) except
25 that the dsDNA was digested with HindIII and BamHI. Clones from pools seven and ten were tested for transcript self-cleavage from linearized plasmids. For clones from Pool 13 plasmid DNA was amplified by PCR, using the RT- and PCR-primers, and then transcribed. Rates of intramolecular cleavage of transcripts were determined as described but with 10 u of enzyme per μ l. Active clones were sequenced. A transcript
30 of only 50 nucleotides, without the extra sequences for cloning beyond stem III, showed a k_{in-cis} of 0.32 min⁻¹.

By "randomized region" is meant a region of completely random sequence and/or partially random sequence. By completely random sequence is meant a sequence

wherein theoretically there is equal representation of A, T, G and C nucleotides or modified derivatives thereof, at each position in the sequence. By partially random sequence is meant a sequence wherein there is an unequal representation of A, T, G and C nucleotides or modified derivatives thereof, at each position in the sequence. A
5 partially random sequence can therefore have one or more positions of complete randomness and one or more positions with defined nucleotides.

Characterization of New self-cleaving ribozyme sequences

The intramolecular cleavage rate of the most active sequence of pool 7 was 0.03 min⁻¹ and that of pool 10 was 0.06 min⁻¹ (Figure 3). Seventy clones from pool thirteen
10 were picked and amplified by PCR. Of these 20 gave full length DNA and were transcribed and of these 14 showed intramolecular cleavage. These 14 active ribozymes could be divided into two groups: one group contained transcripts with 22 nucleotides in the randomized region and the second group contained a deletion in this region with only 21 nucleotides being present. Several sequences were represented twice such as those of
15 clones 29, 36 and 40. One of the most active sequences, from clone 13/40, with k_{in-cis} of 0.52 min⁻¹ was chosen for further study. In comparison, under these conditions the intramolecular cleavage of the native hammerhead with a GUC triplet was k_{in-cis} of 0.56 min⁻¹, indicating that the cleavage activity of the selected ribozyme, Rz13/40, with an AUG triplet is comparable.

20

In order to acquire some information on the secondary structure of Rz13/40 a limited nuclease digest of the 3'-end-labeled, full-length ribozyme was performed. Rz13/40 was prepared by transcription in such a way that cleavage was minimized and full-length ribozyme. A full length transcript for the intramolecularly cleaving ribozyme
25 was generated by transcription at 12°C for 8 h (Frank *et al.*, 1996, *RNA*, 2, 1179; incorporated by reference herein) and 3'-end labeled with ³²pCp. Limited nuclease digestions with RNase A, RNase T1 and nuclease S1 were performed as Hodson *et al.*, 1994, *Nucleic Acids Res.*, 22, 1620; Loria *et al.*, 1996, *RNA*, 2, 551; both are incorporated by reference herein). Alkaline hydrolysis of labeled RNA was performed in 50 mM
30 NaHCO₃ at 100°C for 7.5 minutes.

Limited digestion with RNase A and T1 and nuclease S1, which indicates single-stranded regions, gave a digestion pattern consistent with Rz13/40 adopting a

hammerhead-type structure, which comprised three base-paired helices surrounding a single-stranded core (Fig. 4). Since the structure of Rz13/40 resembles the hammerhead structure, the same numbering system has been adopted, with positions 3 and 9 in the core being considered vacant.

- 5 The data of nuclease digestion corresponded fairly well with the MFOLD structure except for a few discrepancies. MFOLD shows base pairing in the core region between U⁷ and G¹⁴; G⁸ and C¹³; G^{L2.1} and U^{L2.5}; and U⁴ and G¹⁷ although there is cleavage at these positions by the nucleases.

Ribozyme Engineering

- 10 Sequence, chemical and structural variants of ribozymes of the present invention can be engineered using the techniques shown above and known in the art to cleave a separate target RNA or DNA *in trans*. For example, the size of ribozymes can, be reduced or increased using the techniques known in the art (Zaug *et al.*, 1986, *Nature*, 324, 429; Ruffner *et al.*, 1990, *Biochem.*, 29, 10695; Beaudry *et al.*, 1990, *Biochem.*, 29, 6534; McCall *et al.*, 1992, *Proc. Natl. Acad. Sci., USA.*, 89, 5710; Long *et al.*, 1994; Supra; Hendry *et al.*, 1994, *BBA* 1219, 405; Benseler *et al.*, 1993, *JACS*, 115, 8483; Thompson *et al.*, 1996, *Nucl. Acids Res.*, 24, 4401; Michels *et al.*, 1995, *Biochem.*, 34, 2965; Been *et al.*, 1992, *Biochem.*, 31, 11843; Guo *et al.*, 1995, *EMBO. J.*, 14, 368; Pan *et al.*, 1994, *Biochem.*, 33, 9561; Cech, 1992, *Curr. Op. Struc. Bio.*, 2, 605; Sugiyama *et al.*, 1996, *FEBS Lett.*, 392, 215; Beigelman *et al.*, 1994, *Bioorg. Med. Chem.*, 4, 1715; all are incorporated in its totality by reference herein). For example the stem-loop II domain of the ribozymes may not be essential for catalytic activity and hence could be systematically reduced in size using a variety of methods known in the art, to the extent that the overall catalytic activity of the ribozyme is not significantly decreased.

- 25 Further rounds of *in vitro* selection strategies described herein and variations thereof can be readily used by a person skilled in the art to evolve additional nucleic acid catalysts and such new catalysts are within the scope of the instant invention.

Example 2: Trans-cleaving Ribozymes

- 30 The self-cleaving ribozymes can be divided into separate ribozyme and substrate domains to create a functional bimolecular complex. This ribozyme presumably interacts with the substrate domain by forming base-paired regions that are analogous to helices I

and II of the hammerhead ribozyme (Figure 1). Likewise, the substrate specificity of ribozymes can presumably be altered by changing the sequences of the substrate-binding arms to complement the sequence of the desired substrate molecule, as was achieved with the ribozyme from clone 40 self-cleaving RNA (Figure 5).

5 To further characterize the selected ribozyme for intermolecular cleavage, Rz13/40 was divided into a catalytic portion and the corresponding substrate strand (Fig 5). The initial ribozyme construct was designed with 5 bp each in stems I and III for annealing to the substrate. Stems of this length were chosen because they had been reported to give reliable kinetic data with the hammerhead. Cleavage kinetics of intermolecularly
10 cleaving ribozymes was performed with chemically synthesized ribozyme and substrate in 50 mM Tris-HCl (pH 8.0), and 10 mM $MgCl_2$ with 50 to 500 nM ribozyme and 25 nM substrate for single turnover, and 50 to 500 nM substrate with 5 to 25 nM ribozyme for multiple turnover kinetics as described (Hertel *et al.*, *Biochemistry* 33, 3374, 1994). The intermolecular version of Rz13/40 of the present invention gave a very
15 high K_M (1.1 mM) and low k_{cat} (0.1 min^{-1}) under single turnover conditions and was inactive under multiple turnover conditions. As there was no doubt about the intramolecular cleavage of Rz13/40, the lack of catalytic activity under multiple turnover conditions must be associated with the design of the intermolecular version. After analysis of the ribozyme-substrate complex by native gel it was concluded that
20 formation of the complex was inefficient under the conditions used.

A second ribozyme construct was synthesized, where stem III was extended to 10 base-pairs. Native gel analysis confirmed the formation of the ribozyme-substrate complex and the ribozyme cleaved its corresponding substrate efficiently under both
25 single and multiple turnover conditions (Table II). The discrepancy between k_{cat} and k_{cat}' (0.06 and 0.91 min^{-1} respectively) is presumably the result of product inhibition, which is a common effect observed with the native hammerhead. The time course of cleavage was followed for about 12 half-lives under single turnover conditions. Reactions were first order up to 60% cleavage within the first minute and reached a total of 80%. First order end points with the conventional hammerhead are commonly 70 to 75% (19).
30 Steady state cleavage rates were linear for several turnovers. Although the hammerhead and class I ribozymes cleave at different internucleotide linkages (Figure 1), both ribozymes appear to proceed by a similar chemical mechanism. The hammerhead ribozyme is known to produce a 2',3'-cyclic phosphate at the terminus of the 5'-cleavage product, thereby leaving a 5'-hydroxyl terminus on the 3'-cleavage fragment.

The site of substrate cleavage was confirmed as being behind G of the AUG cleavage triplet by comparison of the ribozyme cleavage product, obtained under single turnover conditions for 30 min, with the products of a limited alkaline hydrolysis of the 5'-end-labeled substrate and with a partial RNase T1 digest. Reactions were performed in 10 μ l containing RNA, 50 mM Tris (pH 8) and CNPase (Sigma Chemicals) (0.62 u to 0.012 u/ μ l) and incubation at 45°C for 1.5 h. Product analysis was on a 20% PAGE where the cyclic phosphate-terminated product runs slightly slower than the ring-opened derivative. In addition, the cleavage products were also shown to be the 2', 3'-cyclic phosphate and a 5'-hydroxyl by hydrolysis with CNPase and labeling with T4 PNK respectively. Thus Rz13/40 cleaves in a similar manner to the hammerhead. In order to investigate the properties and sequence requirements of the selected ribozyme Rz13/40, mutations were made on the substrate and ribozyme strands. In the native hammerhead, stem II has been shortened to as few as two base-pair without significant loss in activity. However, the removal of a single base-pair (10.3/11.3) in Rz13/40 resulted in a 1000-fold loss in activity for the cleavage behind an AUG triplet indicating the stem-loop structure has a wider role in catalysis.

The effect of mutations around the cleavage site have also been investigated under single and multiple turnover conditions (Table II). Surprisingly, the AUA triplet was also cleaved quite efficiently even though it was not selected for, again yielding a 2', 3'-cyclic phosphate. Triplets terminating in a uridine or cytidine were either cleaved extremely slowly or not at all. Thus this ribozyme is purine nucleoside specific. Whether this indicates a necessity for base pairing between U⁴ and the nucleotide to be cleaved, as possible with AUG and AUA, is uncertain at present.

As the native hammerhead strictly requires a uridine in the middle of the triplet, it was of interest to test whether this was applicable to Rz 13/40. Interestingly the triplet AAG was cleaved with a k_{cat} of 0.57 min⁻¹ under single turnover conditions, multiple turnover conditions showed a k_{cat} of 0.09 min⁻¹. It was also tested whether the nucleotide next to the cleavage site was of importance. Cleavage of the triplet AUG with a following U or G was found to be only fractionally slower in single turnover than cleavage of AUG-A (Table II).

Inspection of the ribozyme sequences reveals some interesting features. The sequence 5'-GCGCG at positions 11.2 to 14 is present in all ribozymes from pool 10

onwards. This would indicate that this sequence may be an indispensable part for activity just as is the case for the GAA sequence in the conventional hammerhead.

5 Of the two classes of ribozyme isolated in Pool 13, the ribozymes with a deletion in the randomized region are the more active intramolecular cleavers. These ribozymes appear to have an overall secondary structure which is similar to that of the native hammerhead, but there are significant differences.

10 Obvious differences include the 'vacancies' at positions 3 and 9 in the core as well as the altered sequence to positions 12 to 14 already mentioned. More subtle differences are associated with stem-loop II. In the hammerhead a variety of structures are tolerated: the stem can be reduced to two base-pair, and the loop can be of virtually any size and even contain non-nucleotidic linkers. In the selected ribozymes similar to Rz13/40, less variation in the stem-loop structure is observed. In the active ribozymes of this class, stem-loop II consists of two G:C base-pairs adjacent to the core; followed by two A:U base-pairs; and finally terminating in a five nucleotide loop, where variations at only 15 positions L2.4 and L2.5 have been detected. The length of the stem appears to have a large effect on catalytic efficiency, since the removal of a single base-pair (10.3/11.3) abolishes activity. The most active sequences in pool 13 have a fully complementary stem II. One mismatch at 10.2/11.2 as in Rz13/1 approximately halves the activity. It is interesting to note that in Pool 13, the more active ribozymes contain a deletion in the 20 randomized region, which is 21 nucleotides instead of 22. Of these only Rz 13/31 and 13/29 show reasonable activity. One can draw a secondary structure for these with a stem II with two mismatches and the extra nucleotide in the core region.

25 The new triplet specificity should be useful for the application of the ribozyme family of the present invention for the inhibition of gene expression in that it expands the targets on a mRNA for cleavage. Although the conventional NUX triplets might be considered sufficient for a wide application, the accessibility of oligodeoxynucleotides to mRNAs is very restricted. Thus the specificity of the new ribozyme for cleavage at purines, apparently independent of the nature of the neighboring nucleotides, should represent a considerable advantage over the native NUX-cleaving ribozyme for this 30 application.

Diagnostic uses

Enzymatic nucleic acids of this invention may be used as diagnostic tools to examine genetic drift and mutations within diseased cells or to detect the presence of target RNA in a cell. The close relationship between ribozyme activity and the structure of the target RNA allows the detection of mutations in any region of the molecule which alters the base-pairing and three-dimensional structure of the target RNA. By using multiple ribozymes described in this invention, one may map nucleotide changes which are important to RNA structure and function *in vitro*, as well as in cells and tissues. Cleavage of target RNAs with ribozymes may be used to inhibit gene expression and define the role (essentially) of specified gene products in the progression of disease. In this manner, other genetic targets may be defined as important mediators of the disease. These experiments will lead to better treatment of the disease progression by affording the possibility of combinational therapies (e.g., multiple ribozymes targeted to different genes, ribozymes coupled with known small molecule inhibitors, or intermittent treatment with combinations of ribozymes and/or other chemical or biological molecules). Other *in vitro* uses of ribozymes of this invention are well known in the art, and include detection of the presence of mRNAs associated with disease condition. Such RNA is detected by determining the presence of a cleavage product after treatment with a ribozyme using standard methodology.

In a specific example, ribozymes which can cleave only wild-type or mutant forms of the target RNA are used for the assay. The first ribozyme is used to identify wild-type RNA present in the sample and the second ribozyme will be used to identify mutant RNA in the sample. As reaction controls, synthetic substrates of both wild-type and mutant RNA will be cleaved by both ribozymes to demonstrate the relative ribozyme efficiencies in the reactions and the absence of cleavage of the "non-targeted" RNA species. The cleavage products from the synthetic substrates will also serve to generate size markers for the analysis of wild-type and mutant RNAs in the sample population. Thus each analysis will require two ribozymes, two substrates and one unknown sample which will be combined into six reactions. The presence of cleavage products will be determined using an RNase protection assay so that full-length and cleavage fragments of each RNA can be analyzed in one lane of a polyacrylamide gel. It is not absolutely required to quantify the results to gain insight into the expression of mutant RNAs and putative risk of the desired phenotypic changes in target cells. The expression of mRNA whose protein product is implicated in the development of the

phenotype is adequate to establish risk. If probes of comparable specific activity are used for both transcripts, then a qualitative comparison of RNA levels will be adequate and will decrease the cost of the initial diagnosis. Higher mutant form to wild-type ratios will be correlated with higher risk whether RNA levels are compared qualitatively or quantitatively.

Additional Uses

Potential usefulness of sequence-specific enzymatic nucleic acid molecules of the instant invention might have many of the same applications for the study of RNA that DNA restriction endonucleases have for the study of DNA (Nathans *et al.*, 1975 *Ann. Rev. Biochem.* 44:273). For example, the pattern of restriction fragments could be used to establish sequence relationships between two related RNAs, and large RNAs could be specifically cleaved to fragments of a size more useful for study. The ability to engineer sequence specificity of the ribozyme is ideal for cleavage of RNAs of unknown sequence.

Other embodiments are within the following claims.

Table I

Table I: 2.5 μ mol RNA Synthesis Cycle

Reagent	Equivalents	Amount	Wait Time*
Phosphoramidites	65	163 μ L	25
S-Ethyl Tetrazole	23.8	238 μ L	25
Acetic Anhydride	100	233 μ L	5 sec
N-Methyl Imidazole	186	233 μ L	5 sec
TCA	83.2	1.73 mL	21 sec
Iodine	8.0	1.18 mL	45 sec
Acetonitrile	NA	6.67 mL	NA

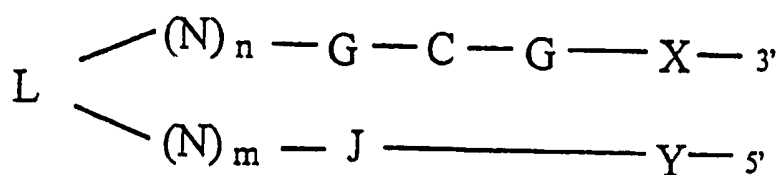
* Wait time does not include contact time during delivery.

Table II. Rate constants for ribozyme 40 cleavage at various triplets with next nucleotide specified. Reaction conditions as in text. la, activity too low for quantitation; na, no activity detectable. MTO, multiple turnover; STO, single turnover

Cleavable Triplet	MTO kinetics		STO kinetics
	k_{cat} (min ⁻¹)	K_M (nM)	k_{cat}' (min ⁻¹)
AUG-A	0.06	50.71	0.91
AUA-A	0.09	24.70	0.34
AUU-A	la	la	0.007
AUC-A	na	na	na
AAG-A			0.57
AUG-U			0.35
AUG-G			0.66

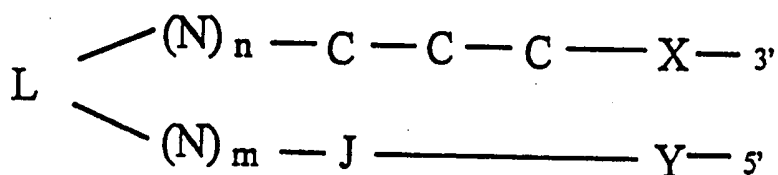
Claims

1. A nucleic acid molecule with an endonuclease activity having the formula I:



- 5 wherein, each N represents independently a nucleotide or a non-nucleotide linker, which may be same or different; X and Y are independent oligonucleotides of length sufficient to stably interact with a target nucleic acid molecule; m and n are integers greater than or equal to 1, wherein if (N)_m and (N)_n are nucleotides, (N)_m and (N)_n may interact by hydrogen bond
 10 interaction; J is an oligonucleotide of length 7 nucleotides; L is a linker which may be present or absent, wherein said linker, when present, is a nucleotide and/or a non-nucleotide linker, wherein said nucleotide linker comprises a single-stranded and/or double-stranded region; _____ represents a chemical linkage; and C and G represent cytidine and guanosine nucleotides,
 15 respectively.

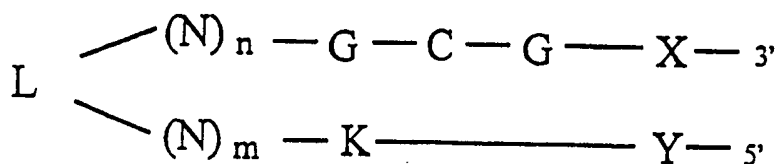
2. A nucleic acid molecule with an endonuclease activity having the formula II:



- 20 wherein, each N represents independently a nucleotide or a non-nucleotide linker, which may be same or different; X and Y are independent oligonucleotides of length sufficient to stably interact with a target nucleic acid molecule; m and n are integers greater than or equal to 1, wherein if (N)_m and (N)_n are nucleotides, (N)_m and (N)_n may interact by hydrogen bond

interaction; J is an oligonucleotide of length 7 nucleotides; L is a linker which may be present or absent, wherein said linker, when present, is a nucleotide and/or a non-nucleotide linker, wherein said nucleotide linker comprises a single-stranded and/or double-stranded region; _____ represents a chemical linkage; and C represent cytidine nucleotide.

3. A nucleic acid molecule with endonuclease activity having the formula III:



wherein, each N represents independently a nucleotide or a non-nucleotide linker, which may be same or different; X and Y are independent oligonucleotides of length sufficient to stably interact with a target nucleic acid molecule; m and n are integers greater than or equal to 1, wherein if $(\text{N})_m$ and $(\text{N})_n$ are nucleotides, $(\text{N})_m$ and $(\text{N})_n$ may interact by hydrogen bond interaction; K is an oligonucleotide of length 6 nucleotides; L is a linker which may be present or absent, wherein said linker, when present, is a nucleotide and/or a non-nucleotide linker, wherein said nucleotide linker comprises a single-stranded and/or double-stranded region; _____ represents a chemical linkage; and C and G represent cytidine and guanosine nucleotides, respectively.

4. The nucleic acid molecule of claim 1, wherein, the sequence of said J in said nucleic acid molecule is a sequence selected from the group consisting of 5'-GGCAUCC-3', 5'-AGCAUU-3', 5'-GCAUCA-3', 5'-AGCAUC-3', and 5'-AGCGUC-3'.
5. The nucleic acid molecule of claim 2, wherein, the sequence of said J in said nucleic acid molecule is 5'-GGAAUC-3'.
6. The nucleic acid molecule of claim 3, wherein, the sequence of said K in said nucleic acid molecule is a sequence selected from the group consisting of 5'-UGAUG-3', 5'-UGAUC-3', and 5'-UGGGC-3'.

7. The nucleic acid molecules of any of claims 1-6, wherein said L in said nucleic acid molecule is nucleotide linker.
8. The nucleic acid molecule of claim 7, wherein said nucleotide linker is a nucleic acid aptamer.
- 5 9. The nucleic acid molecule of claim 8, wherein said aptamer is an ATP aptamer.
10. The nucleic acid molecule of any of claims 1-6, wherein said L is non-nucleotide linker.
11. The nucleic acid molecule of any of claims 1-6, wherein said (N)_m and (N)_n each have a nucleotide sequence.
- 10 12. The nucleic acid molecule of claim 11, wherein said nucleotide sequence of said (N)_m is complementary to the nucleotide sequence of said (N)_n.
13. The nucleic acid molecule of any of claims 1-6, wherein said nucleic acid cleaves a separate nucleic acid molecule.
- 15 14. The nucleic acid molecule of claim 13, wherein said separate nucleic acid molecule is RNA.
15. The nucleic acid molecule of claim 13, wherein said nucleic acid comprises between 12 and 100 bases complementary to said separate nucleic acid molecule.
- 20 16. The nucleic acid molecule of claim 13, wherein said nucleic acid comprises between 14 and 24 bases complementary to said separate nucleic acid molecule.
17. A cell including the nucleic acid molecule of any of claims 1-6.
18. The cell of claim 17, wherein said cell is a mammalian cell.
19. The cell of claim 18, wherein said cell is a human cell.
- 25 20. An expression vector comprising nucleic acid sequence encoding at least one of the nucleic acid molecule of any of claims 1-6, in a manner which allows expression of that nucleic acid molecule.

21. A cell including the expression vector of claim 20
22. The cell of claim 21, wherein said cell is a mammalian cell.
23. The cell of claim 21, wherein said cell is a human cell.
- 5 24. A pharmaceutical composition comprising the nucleic acid molecule of any of claims 1-5.
25. A method for modulating expression of a gene in a plant cell by administering to said cell the nucleic acid molecule of any of claims 1-6.
26. A method for modulating expression of gene in a mammalian cell by administering to said cell the nucleic acid molecule of any of claims 1-6.
- 10 27. A method of cleaving a separate nucleic acid comprising, contacting the nucleic acid molecule of any of claims 1-6 with said separate nucleic acid molecule under conditions suitable for the cleavage of said separate nucleic acid molecule.
28. The method of claim 27, wherein said cleavage is carried out in the presence of a divalent cation.
- 15 29. The method of claim 28, wherein said divalent cation is Mg^{2+} .
30. The nucleic acid molecule of claims 1-6, wherein said nucleic acid is chemically synthesized.
31. The expression vector of claim 19, wherein said vector comprises:
- 20 a) a transcription initiation region;
- b) a transcription termination region;
- c) a gene encoding at least one said nucleic acid molecule; and
- wherein said gene is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.
- 25 32. The expression vector of claim 20, wherein said vector comprises:

- 5
- a) a transcription initiation region;
 - b) a transcription termination region;
 - c) an open reading frame;
 - d) a gene encoding at least one said nucleic acid molecule, wherein said gene is operably linked to the 3'-end of said open reading frame; and

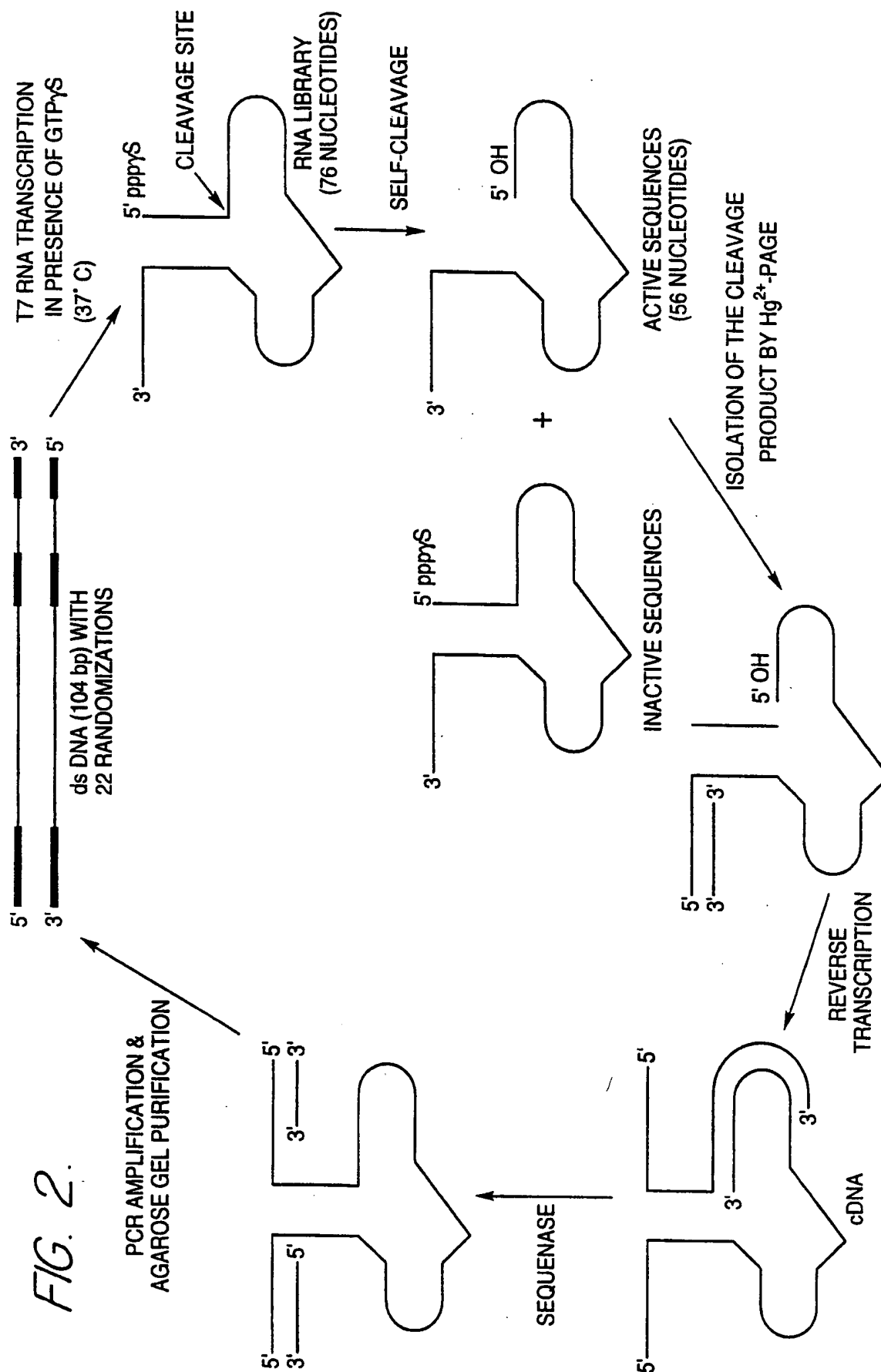
wherein said gene is operably linked to said initiation region, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

- 10
33. The expression vector of claim 20, wherein said vector comprises:
- a) a transcription initiation region;
 - b) a transcription termination region;
 - c) an intron;
 - d) a gene encoding at least one said nucleic acid molecule; and
- 15
- wherein said gene is operably linked to said initiation region, said intron and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

- 20
34. The expression vector of claim 20, wherein said vector comprises:
- a) a transcription initiation region;
 - b) a transcription termination region;
 - c) an intron;
 - d) an open reading frame;
 - e) a gene encoding at least one said nucleic acid molecule, wherein said gene is operably linked to the 3'-end of said open reading frame; and

wherein said gene is operably linked to said initiation region, said intron, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

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FIG. 3.

5' - GGGAGACUGUCUAGAUCAGAGGAGUCUAAUCCU NNNNNN NNNNNNNNNN NNN AUGAUCUGCAGGAUCC - 3'

CLONE NO.		k_{in-cis} (min ⁻¹)	
<i>wt</i> HAMMERHEAD RIBOZYME			
POOL 7	14	CUGAUG AGCGCAAGC GAA	0.66
		GGAUUC AGCCUGACACCGG CCC	0.03
		GGCAUC CCCGGCAUGGUGC GCG	0.06
POOL 10	18	AGCAUU ACCCGGCUUGGUGC GCG	0.20
		GCAUCA CGGGGCAUUCUGC GCG	0.16
		AGCAUC ACCCGGAUGGUGC GCG	0.08
POOL 13		AGCAUC ACCCGGCUUGGUGC GCG	0.08
		AGCGUC CACGGCAUCGAGC GCG	0.04
		UGAUG GCUUGCACUAAGC GCG	0.83
POOL 13	19	UGAUG GCAUGCACUAUGC GCG	0.52
	40 (2x)	UGAUG GCAUGCACUAUGC GCG	0.47
	4	UGAUG GCAUGCACUAUGC GCG	0.33
POOL 13	21	UGAUG GCAUGCACUAUGC GCG	0.24
	1	UGAUG GCAUGCACUAUGC GCG	0.03
	3	UGGGC CGAUCGCAAGGCG GCG	

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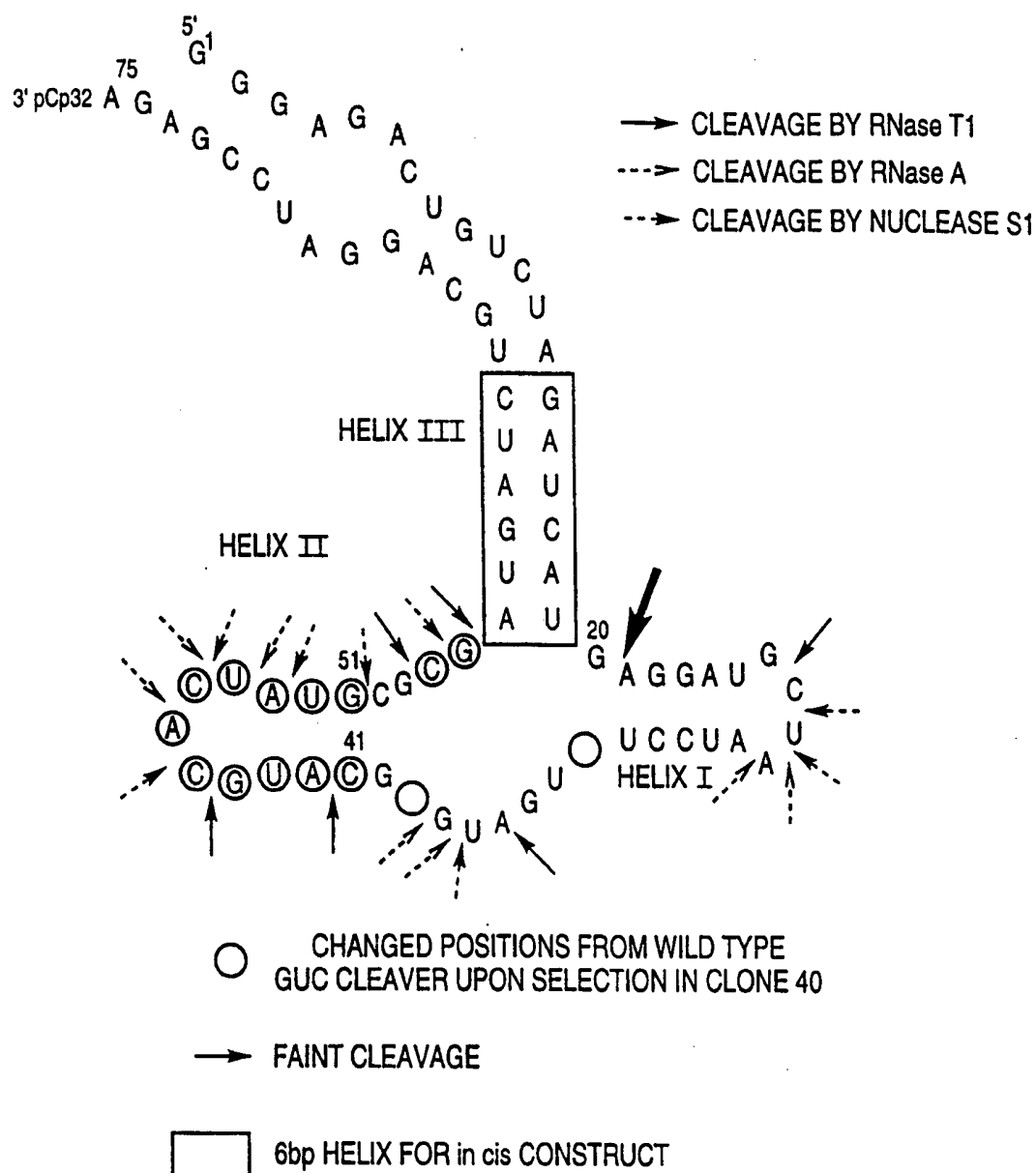
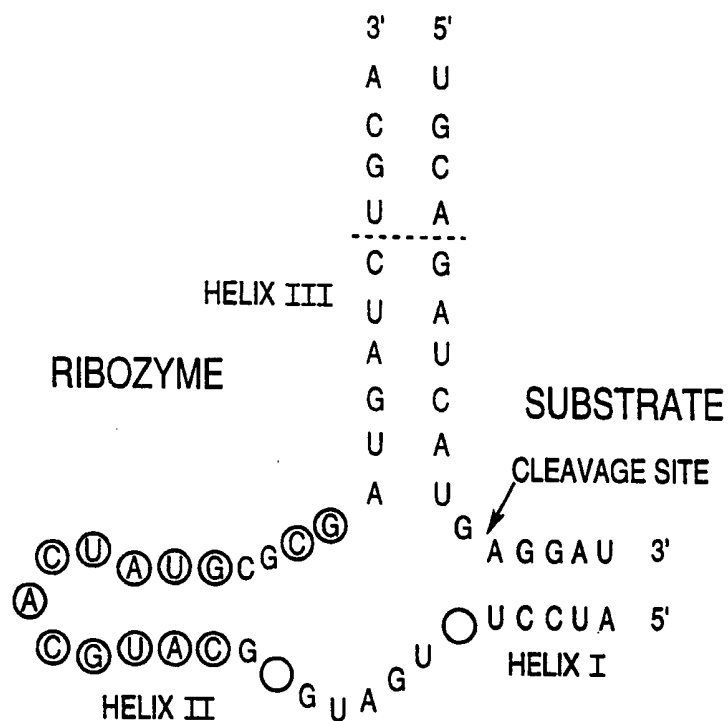


FIG. 4.

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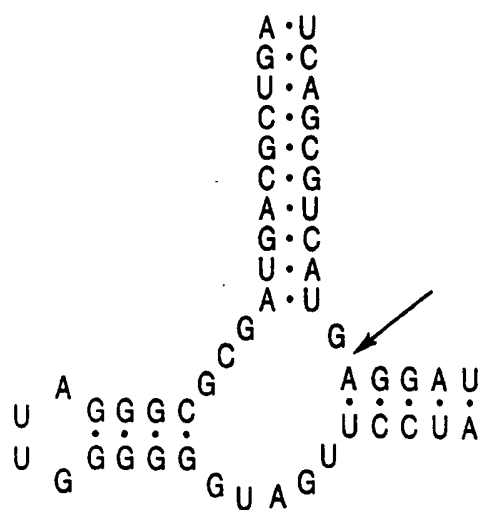


○ CHANGED POSITIONS FROM WILD TYPES
GUC CLEAVER UPON SELECTION IN CLONE 40

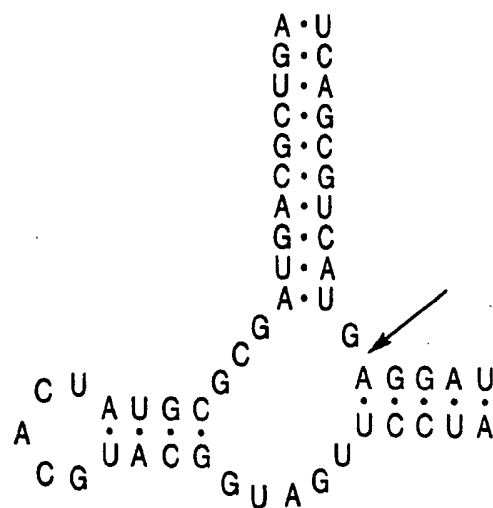
----- BROKEN LINE IDENTIFIES BORDER
OF 6bp CONSTRUCT

FIG. 5.

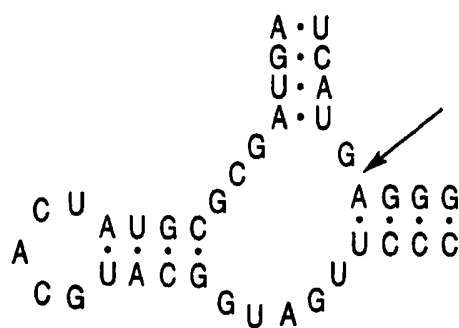
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G-CLEAVING RIBOZYME-A



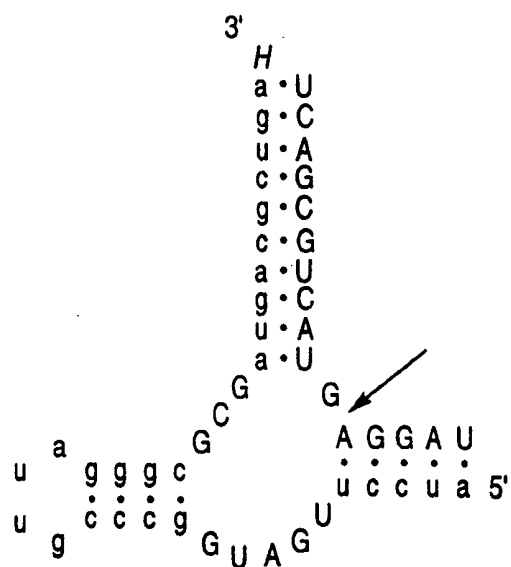
G-CLEAVING RIBOZYME-B



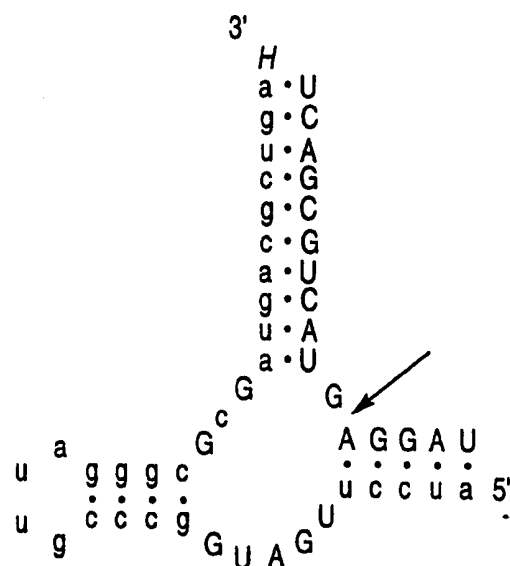
G-CLEAVING RIBOZYME-C

FIG. 6.

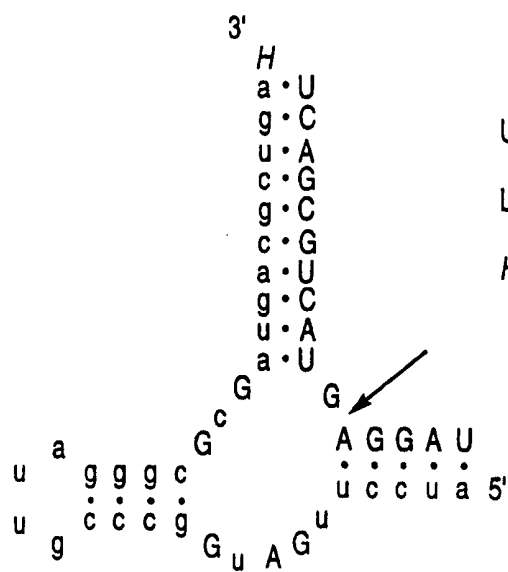
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G-CLEAVING RIBOZYME-A (8-RIBO CORE)



G-CLEAVING RIBOZYME-A (7-RIBO CORE)



G-CLEAVING RIBOZYME-A (5-RIBO CORE)

UPPERCASE = RIBONUCLEOTIDES

LOWERCASE = 2'-O-methyl nucleotides

H = 3'-3' INVERTED ABASIC MOIETY

FIG. 7.